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On Karyomorphs, Cladistics and Taxonomic Status of the *Bufo spinulosus* Species Group (Amphibia: Anura) in Peru

By Jesús H. Córdova, Lima

With 12 figures and 3 tables

Summary

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Keywords: Karyomorphs, karyotypes, cladistics, classification, *Bufo spinulosus* group, Peru.

Resumen

– Sobre cariomorfos, cladística y estado taxonómico de las especies del grupo de *Bufo spinulosus* (Amphibia: Anura) en el Peru –

Se describe los cariotipos bandeados de seis de los ocho taxa reconocidos para el grupo de *Bufo spinulosus* en el Perú. Todos los especímenes exhibieron 2 n = 22 cromosomas (n = 11 bivalentes). Los seis morfo-taxa corresponden a sólo tres cariomorfos: "*spinulosus*", "*limensis*" y "*cophotis*". Se estima que los portadores de cada cariomorfo poseen atributos suficientes para ser considerados especies plenas y en base a un análises cladístico, se propone una nueva clasificación para las especies del grupo *spinulosus* que habitan el Perú.

Zusammenfassung

Die Karyotypen von sechs der acht in Peru nachgewiesenen Taxa der *Bufo spinulosus*-Gruppe werden hier beschrieben. Alle Exemplare besitzen 2 n = 22 Chromosomen (n = 11 Bivalente). Die sechs Morphotaxa entsprechen nur drei verschiedenen Karyomorphen: „*spinulosus*", „*limensis*" und „*cophotis*", wobei die beiden zuerst genannten Formen zueinander ähnlicher sind als *cophotis*. Es wird nachgewiesen, dass jede Karyomorphe ausreichende Merkmale besitzt, um als echte Art angesprochen zu werden. Eine kladistische Analyse wird ausgeführt und eine neue Klassifikation der in Peru vorkommenden Arten der *spinulosus*-Gruppe aufgestellt.

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1. Introduction

Peru is considered one of the twelve megadiverse countries of the world in relation to the number of living species (Lamas 1982, McNeely et alii 1990). This compels us to know with swiftness and precision its natural resources in order to preserve and use them rationally.

At present, the study of the karyotypes or better, the karyomorphs, is an excellent approach for this purpose, since its application exceeds the level of academic or theoretical considerations. It is a fundamental parameter – and sometimes unreplaceable – to solve problems of identification, classification, phylogeny and distribution of the species (Baverstock et alii 1983, 1986, Reig & Uesche 1976, Reig et alii 1980, Volobouev et alii 1987).

Among the most important arguments that are raised to accomplish such studies are: 1) characterization of populations (Dobzhansky 1951, Hsu 1952, Yosida 1981); 2) dependency between chrosomal and genical divergence (Cothran & Smith 1983, Lewontin 1979); and 3) speciation events accompanied by karyotypic changes (Capanna et alii 1977, White 1978, Yosida 1981).

Since cytogenetic studies lead us to the know the species' population structures and their historical relationship (Dobzhansky 1951), it is possible to design appropriate strategies for their conservation and management (Brooks et alii 1992, Morrone & Crisci 1992, Morrone et alii 1996).

Within the specific diversity found in Peru, some sets of species possess characteristics that merit preferential studies. One of them is the Anura order, due to its potential contribution in solving certain problems in areas of human health and nutrition (Córdova 1988, 1991, Paredes et alii 1992).

Among anurans, the genus *Bufo* Laurenti, 1768 stands out, because of its wide world geographical distribution. In Peru, some of its species are relatively frequent, and their role in the control of plant crop pests (Dexter 1932, Lutz 1968, Ochoa 1985), but they are also a good indicator species of environmental pollution (Chakrabarti et alii 1984, Córdova 1988, Córdova et alii 1992, Paredes et alii 1992, Zhuleva & Dubinin 1994, Chuvinishvili 1997).

Of the nearly 380 species included in the family Bufonidae, approximately 210 are placed in *Bufo* (FROST 1985, DUELLMANN & TRUEB 1986, RODRÍGUEZ et alii 1993, POUGH et alii 1998). It is estimated that 46.19 % (97/210) have been cytogenetically studied by conventional staining methods, but only 17.62 % (37) with modern techniques of banding chromosomes (MORESCALCHI 1980, KING 1990, KURAMOTO 1990, CÓRDOVA 1993, CÓRDOVA & DESCAILLEUX 1996, CÓRDOVA et alii 1996).

VELLARD (1959) considered two main *Bufo* species groups as occurring in Peru. The *B. marinus* species group is found in the Tropical Forest region east of the Andes, and in the north coast. The *B. spinulosus* species group is distributed along the Andes, from the north of Peru, south to Chile and Argentina. Because of the size of its distribution, as well as its huge variability in morphology, the *spinulosus* group is considered the most important and complex in Peru (BLAIR 1972).

From the karyomorphic point of view the *spinulosus* group has been poorly studied. In the literature it is only possible to find data originated from conventional staining of populations found outside Peru (BOGART 1972, BRUM-ZORRILLA & SÁEZ 1973, FORMAS 1978, DÍAZ & VELOSO 1979).

Since 1985 I have been reporting data on the karyomorphs of six of the eight taxa that, according to VELLARD (1959), DUELLMANN & OCHOA (1991), and DUELLMANN & SCHULTE (1992), constitute the *spinulosus* group in Peru (CÓRDOVA 1993, CÓRDOVA & DESCAILLEAUX 1985, 1986a, 1986b, 1986c, 1987, 1988, 1989, RODRÍGUEZ et alii 1986). Such information is detailed and discussed in this work, putting emphasis on this phylogenetical and taxonomical implications.

2. Taxonomic problems

This first study on the *spinulosus* group in Peru was made by CORNEJO (1951), while the first taxonomic review was published by VELLARD (1959), who recognized two species, *B. cophotis* Boulenger, 1900 and *B. spinulosus* Wiegmann, 1834, the later including six subspecies, *B. s. spinulosus* Wiegmann, 1834, *B. s. arequipensis* Vellard, 1959, *B. s. flavolineatus* Vellard, 1959, *B. s. limensis* Werner, 1901, *B. s. orientalis* Vellard, 1959 (= *B. limensis vellardi* Leviton & Duellman, 1978) and *B. s. trifolium* (Tschudi, 1845). LUTZ (1968), agreed with VELLARD. BLAIR (1972), MARTIN (1972), CEI (1972) and DUELLMAN (1979) assumed that *spinulosus, flavolineatus, limensis* and *trifolium* were full species, but HARDING (1983), again followed the original view of VELLARD, without including *arequipensis*. SINSCH (1986) considered that a separation at species or subspecies level of the phenotypes *B. spinulosus trifolium* and *flavolineatus* was doubtful, and recognized only one species, *B. spinulosus*. In 1991, DUELLMAN & OCHOA described a new species of *Bufo*, apparently endemic for Peru, from the puna region near Cuzco (Abra Málaga): *B. corynetes*. Tentatively they included it in the *spinulosus* group, as the sister species of *B. variegatus* (Günther, 1870), restricted to the South of Argentina and Chile. Recently, DUELLMAN & SCHULTE (1992) have insisted that *arequipensis, flavolineatus, limensis, spinulosus, trifolium* and *vellardi* (= *limensis vellardi*), are full species.

MAXSON (1984) performed molecular studies in *Bufo*, including three species of the *spinulosus* group from Peru: *spinulosus*, *trifolium* and *flavolineatus*, as well as the subspecies *spinulosus limensis*. In her study there are discrepances between the taxonomic categories assumed and the results obtained. Recently SHERIF (1990, cited by

Sinsch 1991), analyzed 23 enzymatic loci of specimens macromorphologically recognized by Sinsch (1991) as *B. spinulosus* and *B. trifolium*, considering all of them as the same species. Clear disagreement exists on the taxonomic statuses recognized by different authors that have been dealing with the *spinulosus* species group.

3. Material and methods

For this work, 62 specimens of 20 localities in Peru were studied (fig. 1; table 1) which were identified through the key of Vellard (1959). They are deposited in the Museo de Historia Natural of the Universidad Nacional Mayor de San Marcos, Lima, Peru (MUSM 16939–90) and in the Staatliches Museum für Naturkunde, Stuttgart, Germany (SMNS 9250–59).

Cytological preparation was made by cellular suspension of bone marrow and testis tissues, following the procedure cited in Córdova et alii (1987) and Córdova (1993). Colorations done were Conventional (Giemsa to the 2 % in a buffer phosphate, pH 6.8), CBG (Sumner 1972), and Ag-NOR (Kodama et alii 1980), in order to visualize the general chromosomal characteristics, the constitutive heterochromatin distribution (C bands) and the location of the nucleolar organizer regions (NORs), respectively.

The karyotypic-karyomorphic analysis was made with a Leitz-ORTHOPLAN photomicroscope on Agfa ORTHO-25 film and blowups were fixed on bromide paper degrees 2 and 4. The karyotype was determined in all specimens, by counting the chromosomes of at least seven of the better metaphases and/or diacinesis per specimen. The chromosomes were ordered and qualified according to Levan et alii (1964) and Green & Session (1991). To obtain the idyogrames, each morpho-taxon was processed independently, constructing their respective graphs on the basis of their karyometric data (relative length and centromeric index of each chromosomical pair) and integrating into each graph the structural and/or molecular information obtained (location of the C bands and Ag-NORs).

A cladistical analysis using parsimony with Hening 86 software (Farris 1988) in exhaustive options (mhennig* and bb), followed the description of karyomorphs idyograms. The establishment of transformation series the codification of chromosome changes were made in comparison to the idyograms, in order to generate a data matrix for phylogenetic purposes. The criterion for choosing characters was any and all evident chromosomal changes that were shared among taxa and could be polarized using outgroup, following the recommendations of Green (1986) and Borowick (1995) in relation to chromosomal data for phylogenetic analysis (see Appendix). Character states of the outgroup were assigned based on an idyogram constructed using unpublished karyomorphic data of the author, belonging to *Atelopus peruensis* Gray & Cannatella, 1985, a bufonid considered very appropriate for three major reasons: a) it is member of a more basal genus than *Bufo* (Graybeal 1997); b) it is a member of the "primitive" (sensu Lynch, in Lötters & Köhler 1997) *ignescens* group; and c) the species possesses the Tetrodotoxin (TTX), a molecule shared with various skin and body venoms of newts, salamanders, frogs and other toads (Mebs et alii 1995). Derived characters and characters that could not be polarized were not included in the data matrix because their inclusion did not alter the topologies (Borowick 1995).

The following ordered 17 characters were used for analysis of the *Bufo spinulosus* group relationships (between parentheses are the character state assumed):

I. – Duplication of C+ band in telomere of q arm of chromosome 1, found in "*spinulosus*", "*limensis*" and "*cophotis*" (1).

II. – Acquisition of C+ band in telomere of q arm of chromosome 2, found in "*spinulosus*", "*limensis*" and "*cophotis*" (1).

III. – Acquisition of Cm at end of medial third of q arm of chromosome 3, found in "*spinulosus*", "*limensis*" and "*cophotis*" (1).

IV. – Acquisition of conspicuous C+ band in proximal third of p arm of chromosome 3, found in "*spinulosus*" and "*limensis*" (1); this band is loss in "*cophotis*" (2).

V. – Loss the Cm band of telomere of q arm of chromosome 4, found in "*spinulosus*", "*limensis*" and "*cophotis*" (1).

VI. – Loss the C+ band of telomere of p arm of chromosome 5, found in "*spinulosus*", "*limensis*" and "*cophotis*" (1).

Fig. 1. Map of Peru indicating the places of sampling (number, name, coordinates and altitude in meters upon sea level) and the karyomorphs found (symbols): *1.* Cusco (13°30' S, 71°80' E, 3399); – *2.* Paca (11°42' S, 75°30' E, 3390); – *3.* Cieneguilla (12°06' S, 76°48' E, 300); – *4.* Trujillo (08°06' S, 79°01' E, 34); – *5.* Huamachuco (7°48' S, 78°02' E, 3169); – *6.* Canta (11°27' S, 76°27' E, 2819); – *7.* Huancayo (12°03' S, 75°12' E, 3249); – *8.* Ondores (11°04' S, 76°08' E, 4100); – *9.* Huariaca (10°26' S, 76°11' E, 2941); – *10.* Ingenio (11°53' S, 75°15' E, 3460); – *11.* Lima (12°02' S, 77°01' E, 154); – *12.* Corire (16°13' S, 72°28' E, 429); – *13.* Arequipa (16°23' S, 71°32' E, 2335); – *14.* Sabandía (16°27' S, 71°29' E, 2390); – *15.* Camaná (16°37' S, 72°42' E, 96); – *16.* Desaguadero (16°33' S, 69°02' E, 3809); – *17.* Huánuco (09°55' S, 76°14' E, 1894); – *18.* Ambo (10°07' S, 76°12' E, 2064); – *19.* La Viuda (11°35' S, 76°09' E; 4002); – *20.* Casma (09°28' S, 78°18' E, 39). – *Explications: Black triangle* = karyomorph "*spinulosus*"; – *white triangle* = karyomorph "*limensis*"; – *black star* = karyomorph "*cophotis*".

Tab. 1. Quantity and origin of the specimens studied per morfo-taxon (according to VELLARD 1959): *BSS: Bufo spinulosus spinulosus, – BSA: B. s. arequipensis, – BSF: B. s. flavolineatus, – BSL: B. s. limensis, – BST: B. s. trifolium, – BCO: B. cophotis; – F:* females, – *M:* males, – *J/U:* juvenile and/or unidentified sex; – *PT:* partial total per morfo-taxon.

Morpho-Taxon	Sex			PT	Locality
	F	M	J/U		
BSS	6	6	–	12	Desaguadero (Puno), Cuzco (Cuzco), Ondores, Ingenio (Junín).
BSA	12	5	1	18	Sabandía, Arequipa, Camaná, Corire (Arequipa).
BSF	2	3	–	5	Paca, Huancayo (Junín), La Viuda (Junín).
BSL	9	8	3	20	Cieneguilla, Lima (Lima), Camaná (Arequipa), Trujillo (La Libertad), Casma (Ancash).
BST	3	2	–	5	Huánuco, Ambo (Huánuco), Canta (Lima), Huariaca (Pasco).
BCO	1	1	–	2	Huamachuco (La Libertad).
Total				62	

VII. – Loss the Cm intestitiales bands of p and q arms of chromosome 6, found in *"spinulosus"*, *"limensis"* and *"cophotis"* (1).
VIII. – Pericentric inversion in chromosome 6, found in *"spinulosus"* and *"limensis"* (1), no pericentric inversion in *"cophotis"* (0).
IX. – Intesticial deletion of p arm of chromosome 7, found in *"spinulosus"*, *"limensis"* and *"cophotis"* (1).
X. – No presence of RONs in chromosome 7 of *"spinulosus"* (0), presence (by translocation) of RONs in p arm of chromosome 7 in *"limensis"* (1), and presence (by pericentric inversion) of RONs in q arm of chromosome 7 in *"cophotis"* (2).
XI. – Pericentric inversion in chromosome 8, found in *"spinulosus"* and *"limensis"* (1), no pericentric inversion in *"cophotis"* (0).
XII. – Pericentric inversion in chromosome 9, found in *"spinulosus"*, *"limensis"* and *"cophotis"* (1).
XIII. – Acquisition of an interticial c+ band in chromosome 9, found in *"spinulosus"* and *"cophotis"* (chromosome 8 of karyomorph the later) (1); loss in *"limensis"* (2).
XIV. – Pericentric inversion put the RONs in p arm of chromosome 11 in *"spinulosus"* (1); RONs is lost (by translocation) in *"limensis"* and *"cophotis"* (2).
XV. – Acquisition of a Cm band in telomere of p arm in chromosome 1, found in *"spinulosus"* and *"limensis"* (1); absent in *"cophotis"* (0).
XVI. – Acquisition of Cm band in distal third of p arm of chromosome 1, found in *"spinulosus"* and *"limensis"* (1); absent in *"cophotis"* (0).
XVII. – Acquisition of Cm band in distal third of q arm in chromosome 1, found in *"spinulosus"* and *"limensis"* (1); absent in *"cophotis"* (0).

4. Karyomorphic description

All individuals of the sample possess a diploid number 2 n = 22 in bone marrow cells (figs. 2–8). In males, 11 bivalents were found (fig. 9).

As observed in conventional coloration, patterns of C bands, and location of secondary constrictions, the morpho-taxa *spinulosus, arequipensis, flavolineatus* and *trifolium* show the same karyotype-karyomorph. On the other hand, *limensis* and *cophotis* exhibit conspicuous and constant karyomorphic differences with respect to the previous group.

4.1. Karyomorphs of *Bufo spinulosus* Wiegmann, 1834, *B. arequipensis* Vellard, 1959, *B. flavolineatus* Vellard, 1959 and *B. trifolium* (Tschudi, 1845)

4.1.1. Conventional coloration

The eleven chromosomal pairs can be classified in three groups. The first five comprise a group of large chromosomes, being pairs 1, 2, 3 and 5, metacentrics (m) and pair 4 a submetacentric (sm); pair 6 is a submetacentric chromosome and the only representative of the group of the mid-size chromosomes, and five pairs form the group of small chromosomes, with pairs 8, 9, 10 and 11 being metacentric and pair 7 subtelocentric (st). Pair 11 presents secondary constrictions on the short arm (figs. 2–5, 10 and table 2).

4.1.2. CBG banding

All chromosomal pairs possess C positive bands (C+) in the centromeric regions. Furthermore, a very notable C+ band in the region adjacent to the centromere was found in the short arm (p) on pair 3. There are another C+ band in the long arm (q) of pair 9, in the region near the centromere and on telomeres of q of pair 2. There are also C bands of minor tonality (Cm), in the region adjacent to the centromere and distal third of q as well as in the distal third and telomere of p in pair 1. Also in the distal third of q of pair 3 (figs. 2b–5b and 10).

4.1.3. Ag-NOR staining

The NORs were located in the short arm of pair 11, in the same location of its secondary constrictions (fig. 8).

From now on this karyomorph will be called "*spinulosus*".

4.2. Karyomorph of *Bufo limensis* Werner, 1901

4.2.1. Conventional coloration

The first five large pairs, 1, 2, 3 and 5 are m, and pair 4 is sm; the mid-size chromosome (pair 6) and the five small pairs, all are m. The short arm of pair 7 presents evident secondary constrictions (figs. 6, 11 and table 2).

4.2.2. CBG banding

All chromosomal pairs present C+ bands in the centromeric region. They also present a very notable C+ band in p of pair 3, in the region adjacent to the centromere. Another C+ band is found in telomere of q of pair 2. There are Cm bands in region adjacent to the centromere and distal third of q as well as in the distal third and telomere of p in pair 1. Also in the distal third of q of pair 3 (figs. 6b and 11).

4.2.3. Ag-NOR staining

The NORs were located in the short arm of pair 7, being in the same location of its secondary constrictions (fig. 8b).

From now on this karyomorph will be called "*limensis*".

Tab. 2. Karyometry and chromosomic denomination of the six ingroup morfo-taxa studied for the present work. – *BSS: Bufo spinulosus spinulosus; BSA: B. s. arequipensis; BSF: B. s. flavolineatus; BST: B. s. trifolium; BSL: B. s. limensis; BCO: B. cophotis; LR:* Relative Length; *IC:* Centromeric Index; *RC:* Centromeric Ratio; *TC:* Chromosome Type.

		Chromosome pair No.										
		1	2	3	4	5	6	7	8	9	10	11
BSS	LR	17.423	16.606	14.904	12.413	10.708	7.723	4.803	4.644	4.203	3.532	3.223
	IC	0.472	0.437	0.443	0.364	0.473	0.365	0.304	0.476	0.459	0.453	0.414
	RC	1.145	1.333	1.316	1.725	1.112	1.724	2.627	1.180	1.267	1.205	1.447
	TC	m	m	m	sm	m	sm	sm	m	m	m	m
BSA	LR	16.967	16.812	14.479	13.304	10.404	7.892	4.856	4.611	4.517	3.664	2.555
	IC	0.484	0.424	0.424	0.353	0.489	0.373	0.327	0.453	0.454	0.497	0.400
	RC	1.122	1.367	1.384	1.919	1.085	1.733	1.723	1.614	1.282	1.237	1.361
	TC	m	m	m	sm	m	sm	sm	m	m	m	m
BSF	LR	18.586	17.423	13.788	11.945	10.195	8.414	4.874	4.753	4.023	3.132	2.932
	IC	0.484	0.420	0.445	0.334	0.473	0.355	0.271	0.462	0.462	0.408	0.384
	RC	1.151	1.357	1.283	2.036	1.149	1.893	1.690	2.582	1.296	1.764	1.123
	TC	m	m	m	sm	m	sm	sm	m	m	m	m
BST	LR	17.611	17.312	14.630	12.503	10.601	7.166	4.900	4.714	4.501	3.243	2.845
	IC	0.478	0.422	0.441	0.309	0.474	0.356	0.282	0.443	0.480	0.453	0.386
	RC	1.124	1.374	1.401	2.035	1.126	1.823	2.633	1.191	1.181	1.275	1.726
	TC	m	m	m	sm	m	sm	sm	m	m	m	m
BSL	LR	17.034	15.638	14.289	12.355	10.442	7.885	5.984	4.872	4.566	3.862	3.103
	IC	0.471	0.445	0.453	0.366	0.483	0.453	0.383	0.414	0.445	0.485	0.476
	RC	1.136	1.312	1.221	1.612	1.095	1.236	1.735	1.470	1.323	1.144	1.123
	TC	m	m	m	sm	m	m	m	m	m	m	m
BCO	LR	17.607	16.479	13.714	11.943	10.432	8.562	5.673	4.901	4.162	3.644	2.927
	IC	0.436	0.391	0.383	0.321	0.488	0.461	0.230	0.394	0.419	0.422	0.277
	RC	1.343	1.592	1.654	2.189	1.262	1.199	4.167	1.731	1.533	1.390	2.883
	TC	m	m	m	sm	m	m	st	m	m	m	sm

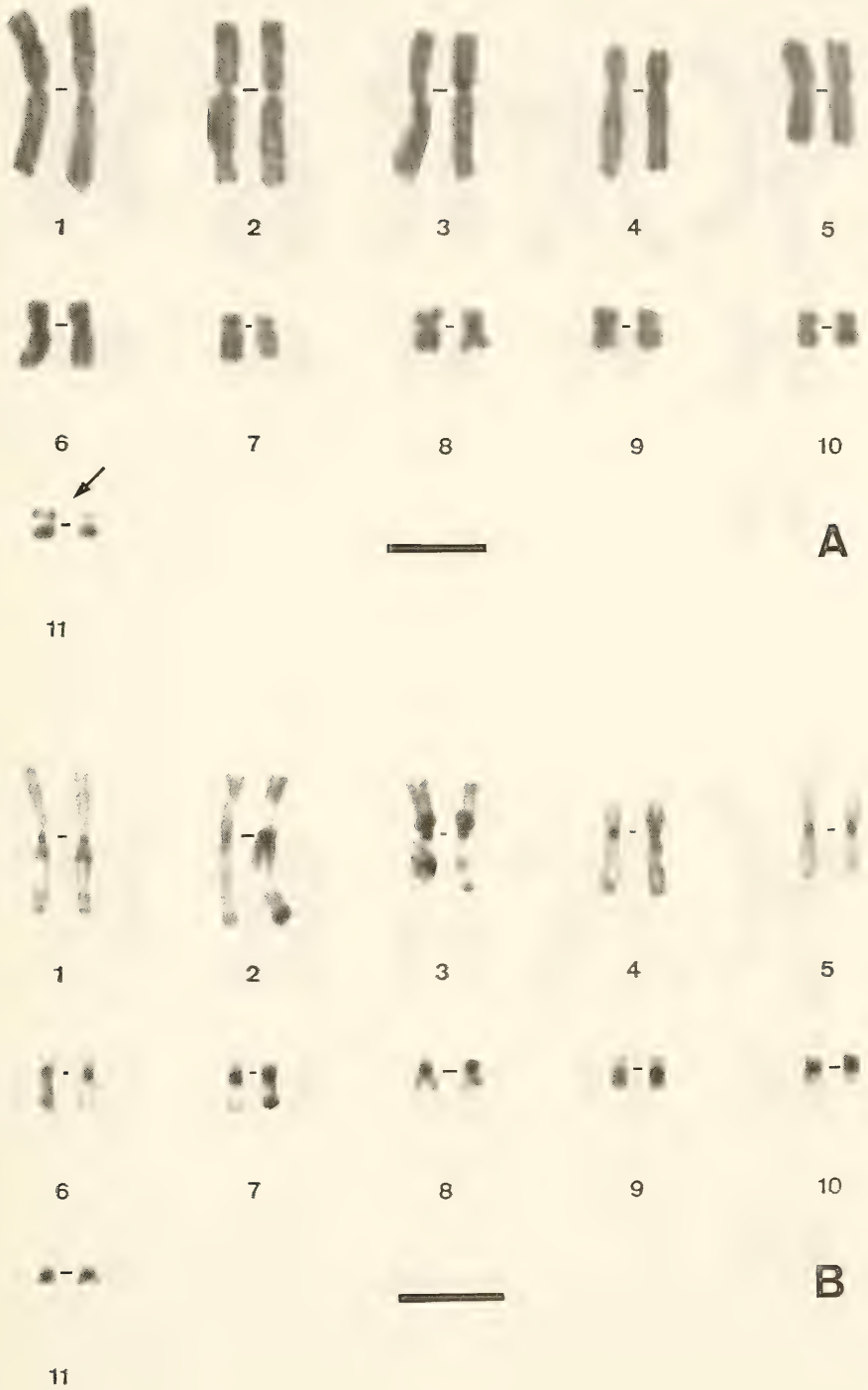

Fig. 2. Karyomorphs of the morpho-taxon *spinulosus* (= "*spinulosus*") in colorations. – A) Conventional; – B) C bands (CBG). – *Arrow* points to a secondary constrictons chromosome carrier. – Bar for Figs. 2–9 = 5 µm.

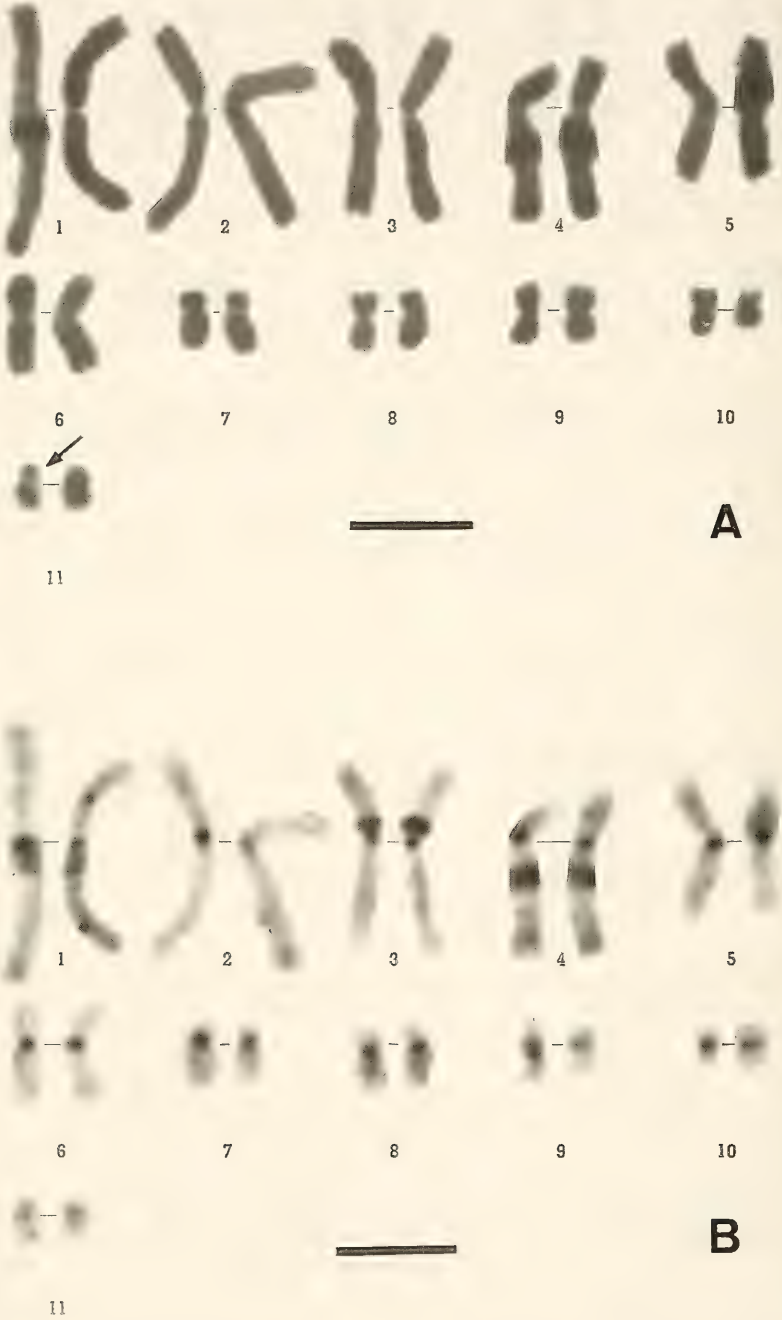

Fig. 3. Karyomorphs of the morpho-taxon *arequipensis* (= "*spinulosus*") in sequential colorations. – A) Conventional; – B) CBG. – *Arrow* points to a secondary constrictons chromosome carrier.

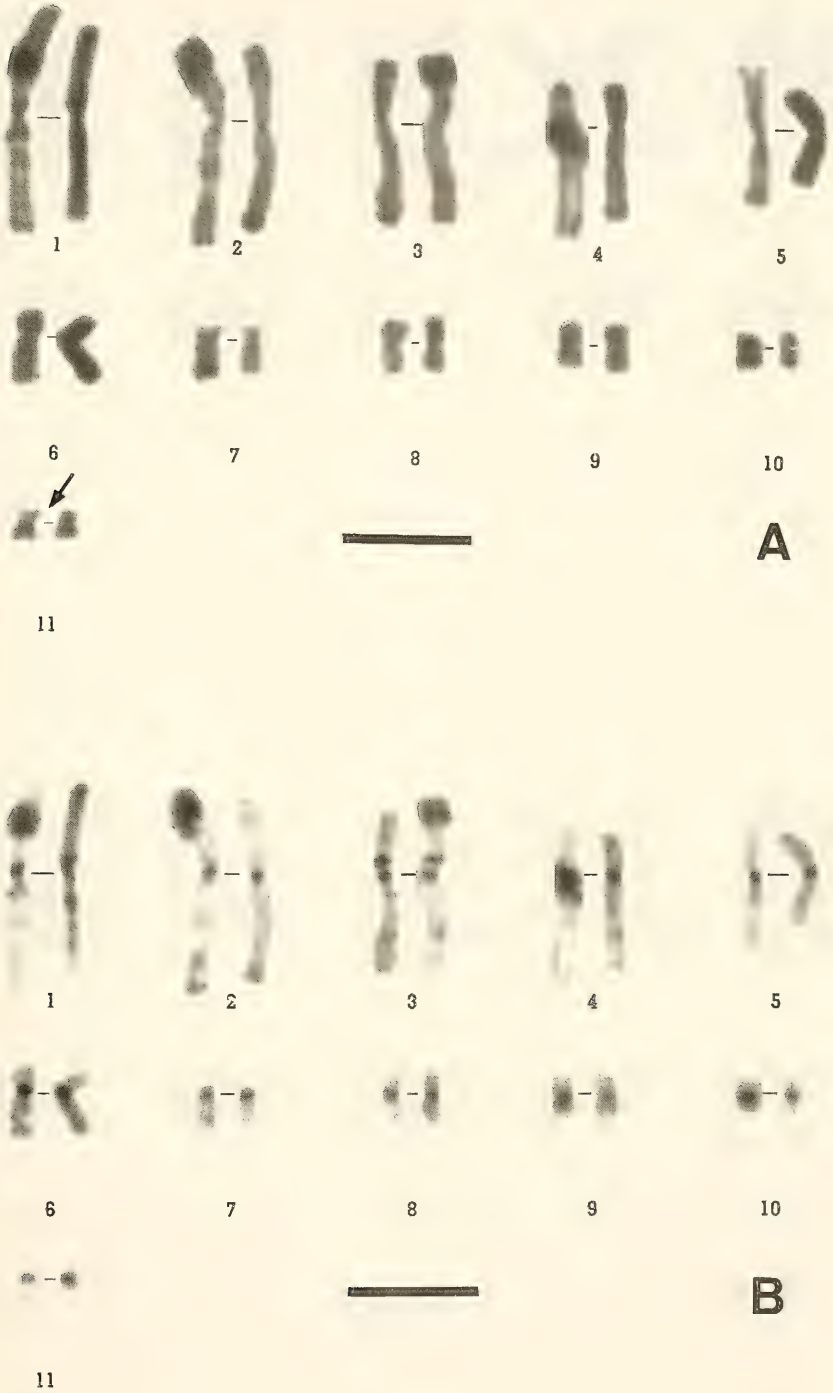

Fig. 4. Karyomorphs of the morpho-taxon *flavolineatus* (= "*spinulosus*") in sequential colorations. – A) Conventional; – B) CBG. – *Arrow* points to a secondary constrictons chromosome carrier.

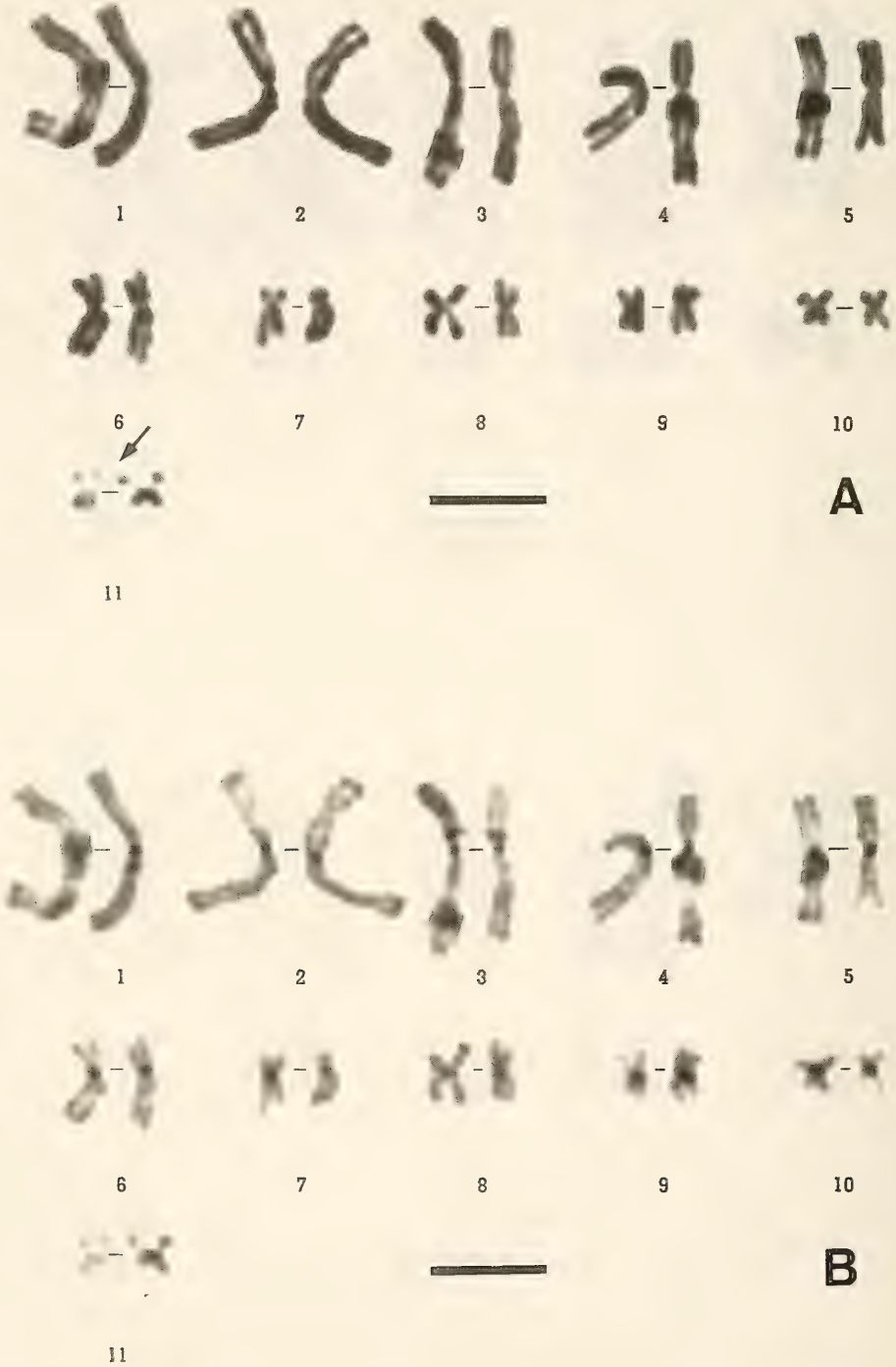

Fig. 5. Karyomorphs of the morpho-taxon *trifolium* (= "*spinulosus*") in sequential colorations. – A) Conventional; – B) CBG. – *Arrow* points to a secondary constrictons chromosome carrier.

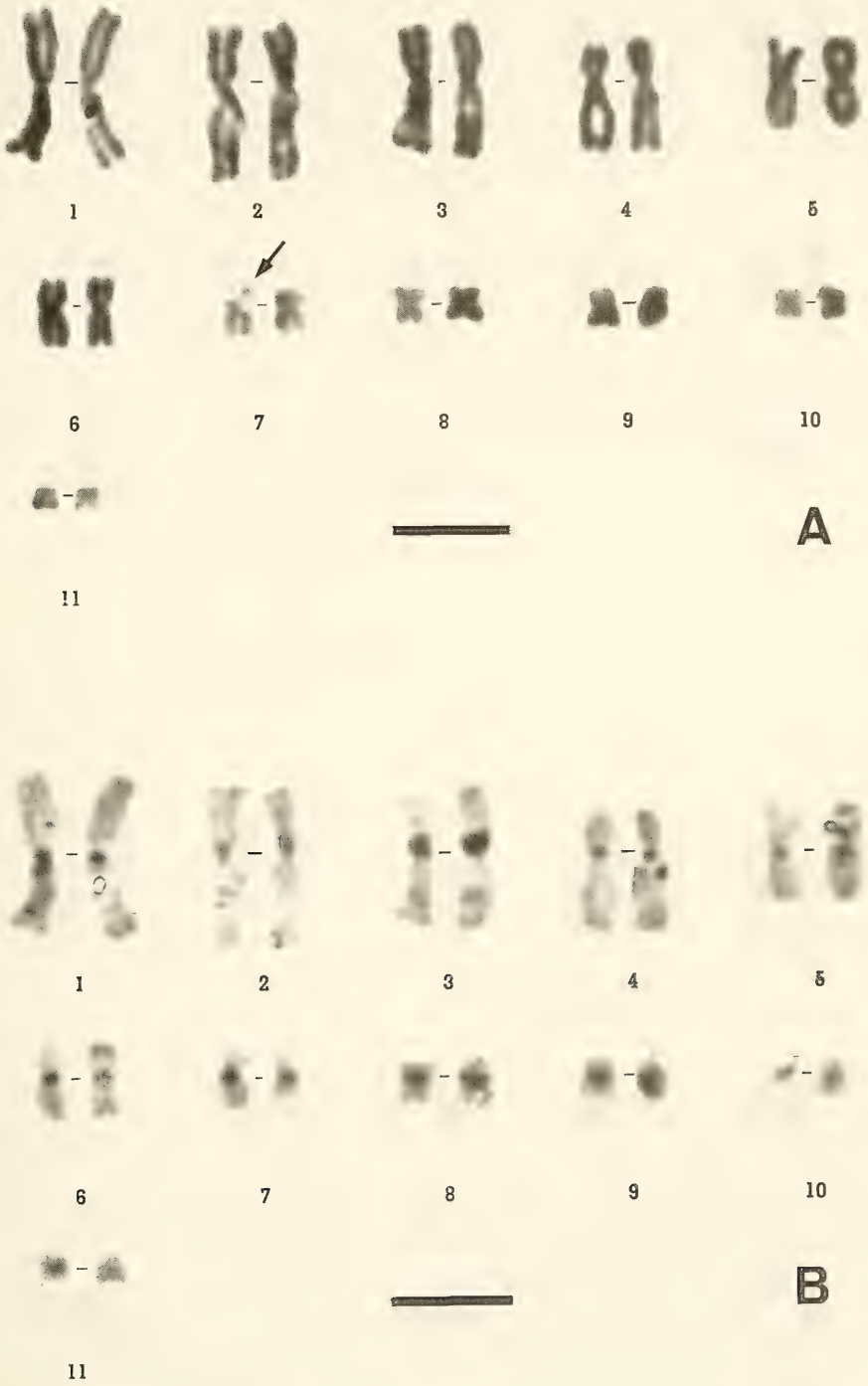

Fig. 6. Karyomorphs of the morpho-taxon *limensis* (= "*limensis*") in sequential colorations. – A) Conventional; – B) CBG. – *Arrow* points to a secondary constrictons chromosome carrier.

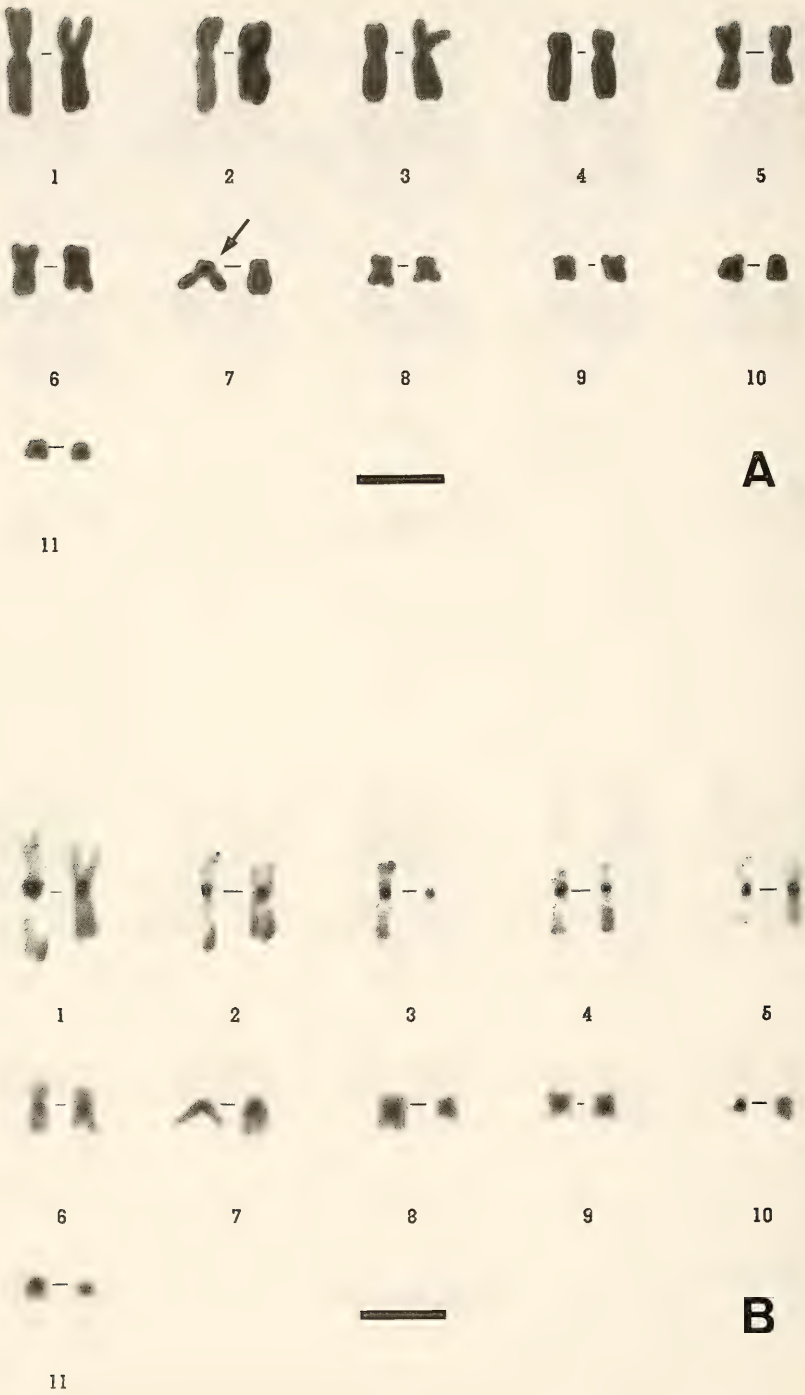

Fig. 7. Karyomorphs of the morfo-taxon *cophotis* (= "*cophotis*") in sequential colorations. – A) Conventional; – B) CBG. – *Arrow* points to a secondary constrictons chromosome carrier.

Fig. 8. Karyomorphs "*spinulosus*" (A) and "*limensis*" (B) colored by Silver Staining procedure (Ag-NOR) to visualize the nucleolar organizer regions *(arrows)*.

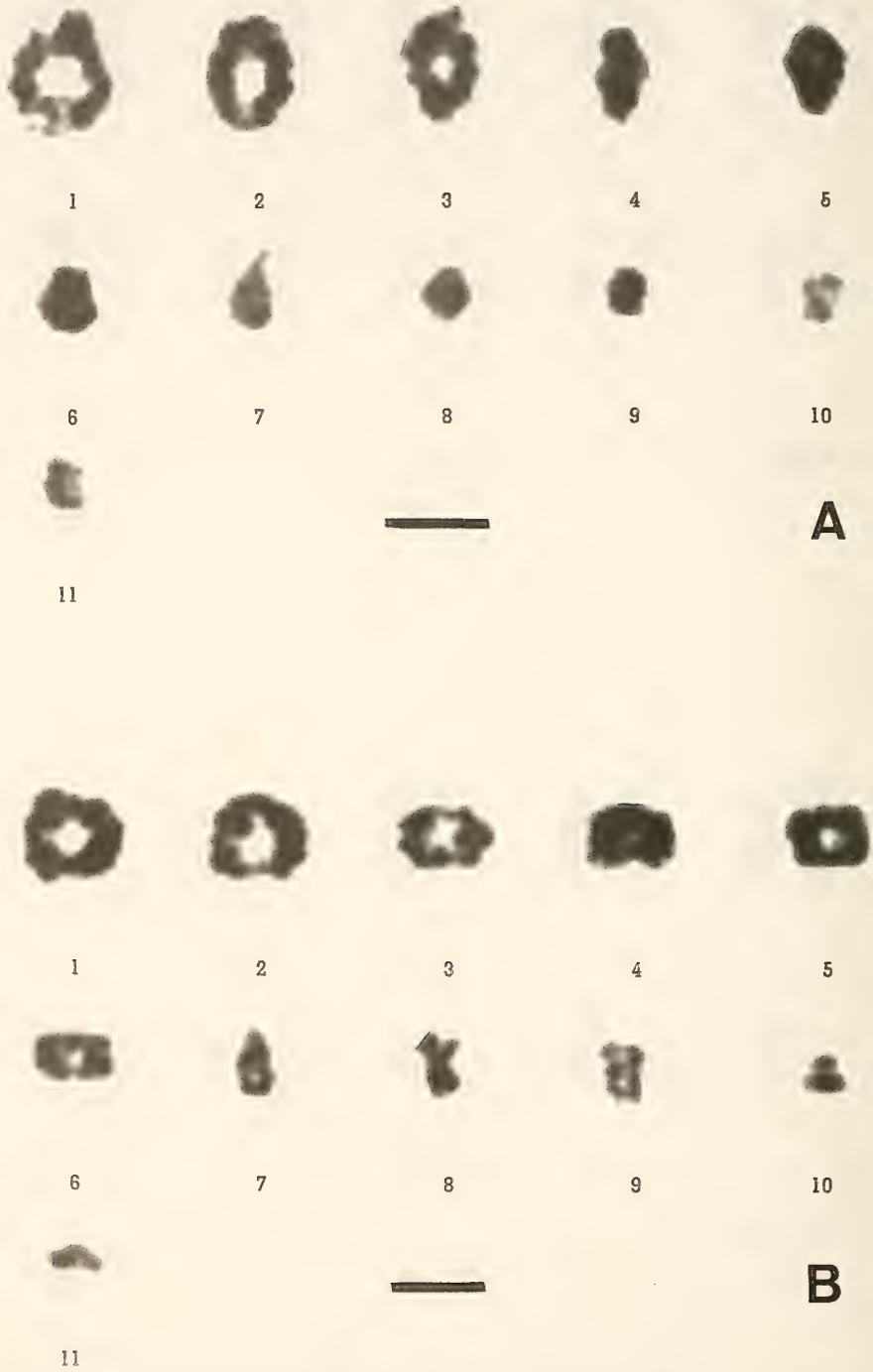

Fig. 9. Bivalents in diacinesis ordered by length. – A) Morpho-Taxon "*spinulosus*"; – B) morpho-taxon *limensis*.

4.3. Karyomorph of *Bufo cophotis* Boulenger, 1990

4.3.1. Conventional coloration

The first five large pairs, 1, 2, 3 and 5 m, and the pair 4 sm; the mid-sized (pair 6) is m, and the five small pairs, 8, 9 and 10 are m; pair 7 is st and 11 is sm. Pair 7 possess as visible secondary constrictions on the long arm, in the region adjacent to the centromere (figs. 7, 11 and table 2).

4.3.2. CBG banding

All chromosomal pairs possess C+ bands in their centromeres. Also exist C+ bands in p of pair 1, in the region adjacent to the centromere, in the proximal third of q of the pair 7, and in the region near to centromere of q of pair 8. Cm bands on telomeres of the long arms of the pairs 1, 2, 3 and 5 was found, as well as a very tenuous one in the distal third of q of pair 3 (figs. 7b and 11).

From now on this karyomorph will be called "*cophotis*".

5. Comparison of the karyomorphs "*spinulosus*", "*limensis*" and "*cophotis*"

The results indicate that the six taxa examined in the present study can be grouped into three karyomorphs. Thus, *spinulosus, arequipensis, flavolineatus* and *trifolium,* share the same karyotype-karyomorph, given that no meaningful differences were found between them. The other two karyomorphs correspond to *limensis* and *cophotis,* clearly distinguishables to the former.

In conventional coloration, the "*spinulosus*" karyomorph differs from "*limensis*" and "*cophotis*" mainly in the location of the secondary constrictions. In "*spinulosus*" it is found in the short arm (p) on pair 11, while in "*limensis*" it is found in the short arm of pair 7, and in "*cophotis*" in the same pair, but in its long arm (figs. 2–7).

The karyometric data indicate small differences related to the centromeric index of some pairs. In "*spinulosus*" chromosomic pair 6 is sm, while in "*limensis*" and "*cophotis*" it is m. Pair 7 is sm in "*spinulosus*", while it is m in "*limensis*" and st in "*cophotis*". Finally, pair 11 is m in "*spinulosus*" and "*limensis*", while in "*cophotis*" it is sm (figs. 2–7, 10, 11 and table 2).

In C banding, "*spinulosus*", "*limensis*" and "*cophotis*" show some differences. The first two karyomorphs diverge notably form "*cophotis*" in the distribution of C bands in pairs 1, 3 and 7. In pair 1, "*spinulosus*" and "*limensis*" possess a Cm band in q, while in "*cophotis*" it occurs in p of the same chromosome, but it is C+. In pair 3, "*spinulosus*" and "*limensis*" possess a C+ band vary notable in p, while it is absent in "*cophotis*". In q of pair 7 of "*cophotis*", there is a C+ band that is not found in "*spinulosus*" and "*limensis*". On the other hand, "*cophotis*" and "*spinulosus*" have the same C band pattern in pair 9, while "*limensis*" lacks the C+ band that is found in q of this pair (figs. 2b–7b, 10 and 11).

The Ag-NOR staining was applied to karyomorphs "*spinulosus*" and "*limensis*", making possible to assert that the NORs are coincident with determined places of secondary constrictions of such karyomorph (in the short arm of pair 11 in the case of "*spinulosus*", and in the same arm but of pair 7 in "*limensis*") (fig. 8a–b).

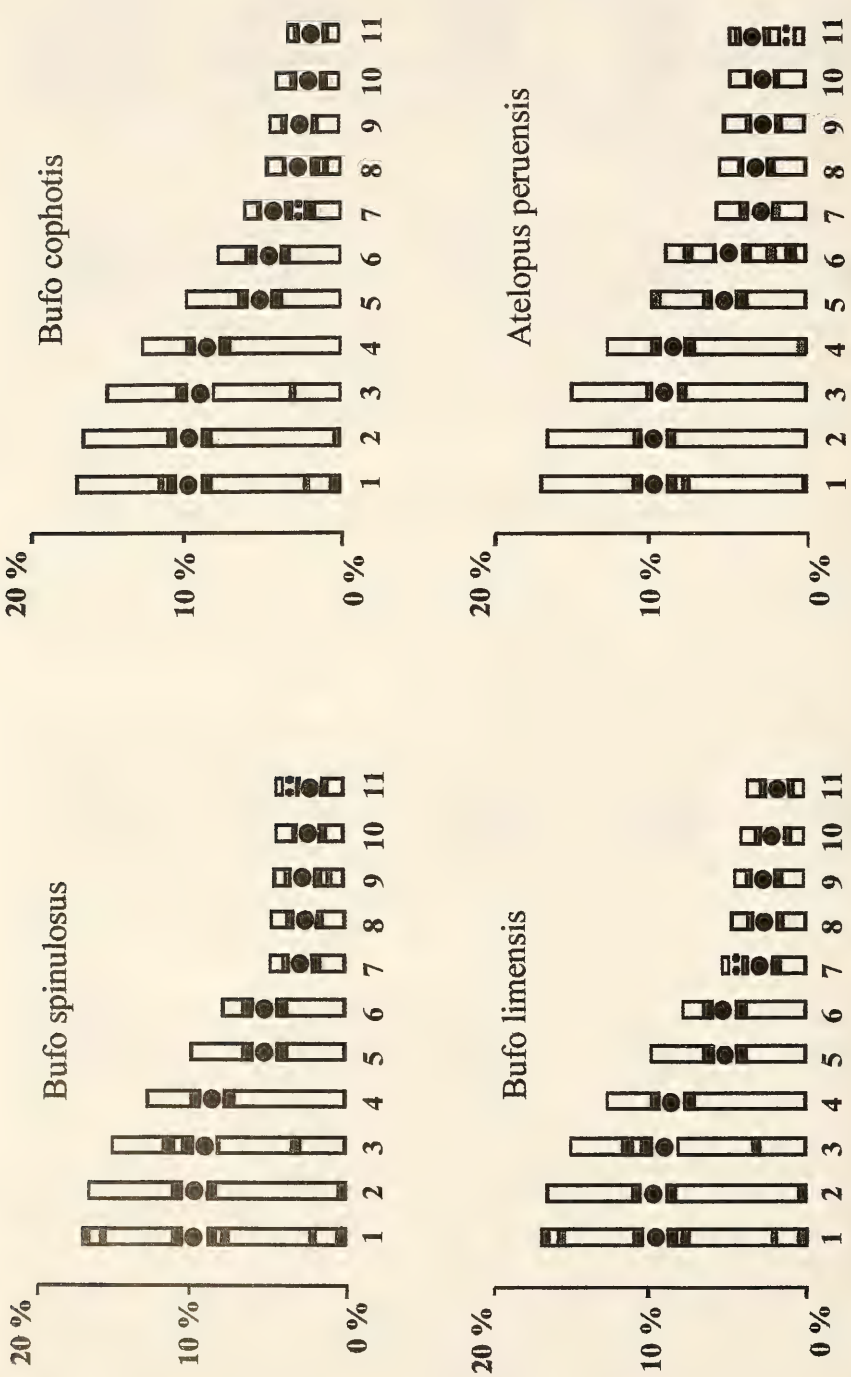

Fig. 10. Integral banding idyogramas of the ingroup morfo-taxa of the present study and of the outgroup *Atelopus peruensis*. – *Double circles* indicate the location of the Ag-NORs.

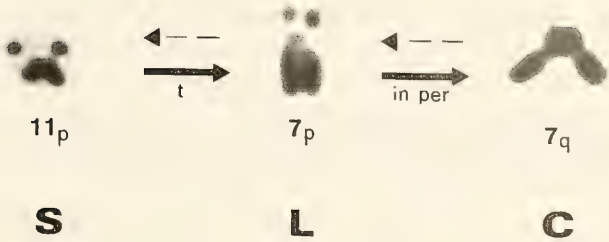

Fig. 11. Evolutionary sequence of the chromosomes bearing of the secondary constrictions (= NORs). – *Explications: Continuous arrows* indicate the more probable sequence, the *interrupted arrows,* the less probable; *S*: "spinulosus", *C*: "cophotis"; *t*: translocation, *in per:* pericentric inversion.

Tab. 3. Data matrix of four taxa [APE (*Atelopus peruensis,* outgroup) *BSS, BSL,* and *BCO*] considered herein versus the seventeen characters *(CHR)* considered for phylogenetic studies.

	Taxa			
	OG	Ingroup		
CHR	APE	BSS	BSL	BCO
I	0	1	1	1
II	0	1	1	1
III	0	1	1	1
IV	0	1	1	2
V	0	1	1	1
VI	0	1	1	1
VII	0	1	1	1
VIII	0	1	1	0
IX	0	1	1	1
X	0	0	1	2
XI	0	1	1	0
XII	0	1	1	1
XIII	0	1	2	1
XIV	0	1	2	2
XV	0	1	1	0
XVI	0	1	1	0
XVII	0	1	1	0

6. Cladistic results

Analysis of the data matrix of seventeen characters (table 3) resulted in one most parsimonious tree with length of 23 steps, a consistence index of 0.91 and a retention index of 0.71 (fig. 12). Eight derived characters (I, II, III, V, VI, VII, IX, and XII) support the monophyly of the *Bufo spinulosus* group, and six support the cluster "*limensis*" (BSL)-"*spinulosus*" (BSS) (characters IV, VIII, XI, XV, XVI and XVII). The "*cophotis*" (BCO) karyomorph is the more basal.

7. Discussion

7.1. Cytophylogenetic interpretation of karyomorphic differences

The discrepancies between the three karyomorphs in conventional coloration can be grouped in: a) location of secondary constrictions-AgNORs; b) centromere position in some pairs; and c) changes in C banding pattern.

a) In the three karyotypes, the different locations of the secondary constrictions could be explained in the following way. One chromosome 11 bearing a secondary constriction in its short arm ("*spinulosus*" type), could have suffered the translocation of this structure towards the short arm of chromosome 7, forming one of the "*limensis*" type. Then such chromosome, could have suffered a pericentric inversion that located the secondary constriction on its long arm, being generating a chromosome of "*cophotis*" type (fig. 11) (a special derived feature). The sequence of events would be different, but findings in *Pedostibes* (Borneo and Sumatra) and *Melanophryniscus* (Argentina) (Bogart 1972, Schmid 1978), all belonging to the Bufonidae, as well as geographically distant members of *Bufo*, such as *B. parvus* (Southeast Asia), *B. mauritanicus* (Africa), and *B. calamita* (Europe) that possess the secondary constrictions on the chromosome 11 (Schmid 1978), make it possible that this could be the ancestral condition and this allows to accept the initial sequence as the most probable, based on parsimony and corological progression (Hennig 1968, Wiley 1980, Cracraft 1983, Maddison et alii 1984).

b) The different position of the centromere in some chromosomal pairs of compared karyomorphs, leads to changes in designation of chromosomal type of the pairs involved. Thus, in the "*spinulosus*" karyomorph, it is possible to find a chromosome 6 sm, while in "*limensis*" and "*cophotis*" karyomorphs it is m; chromosome 7 is sm in "*spinulosus*", m in "*limensis*", and st in "*cophotis*". Finally, chromosome 11 is m in "*spinulosus*" and "*limensis*", and sm in "*cophotis*".

The case of the chromosome 6 can be explained if the existence of a pericentric inversion is assumed. The problem of the morphology of the chromosomes 7 and 11 is related to the presence and location of the secondary constrictions-AgNORs, explained in a). In "*cophotis*", the sm chromosome 11 would be the result of a pericentric inversion, from an m chromosome of "*limensis*" type.

c) The changes in the C bands patterns, between the "*spinulosus*" "*limensis*" and "*cophotis*" karyomorphs, with exception of chromosome 1, could be explained through processes of loss – addition of heterochromatic material. The case of chromosome 1 requires at least an additional pericentric inversion and a process that allows to go from a Cm band in the q arm of "*spinulosus-limensis*" type, to a C+ band in the p arm, as in "*cophotis*" (a more derived character of this transformation series and an autapomorphy for the latter).

Taken as a whole, the chromosomal structural differences found, make the "*cophotis*" karyomorph very different from the "*spinulosus-limensis*" karyomorphs.

7.2. Cladistic inferences

The results of the cladistic analysis obtained at present state of the data ingroup are clear. The first inference is related to the strong support of the monophyly of the

Bufo spinulosus group (eight derived features) and this inference is concordant with previous phenetic approximations (BLAIR 1972, CEI 1972, DUELLMAN & SCHULTE 1992). A second inference is related to the reasonable support for the monophyly of the clusters "*spinulosus-limensis*". Additional karyomorphic data of more species (outgroups and ingroups) or populations from different localities – in and out of Peru – in the near future, will allow us the precise phylogeny for each natural entity of this important species group.

Some authors have critized the use of C-bands in phylogenetic reconstructions due to potential non-homology of these structures at genetic level (BAKER et alii 1987, BOROWICK 1995). In my view, these critiques (or prejudices) violate phylogenetic assumptions, because there exists the same uncertainty for absolute homology in any other type of chromosomal band (or any character) a priori. Homology must be assumed always in absence of real evidence to the contrary (HENNIG 1966). On the other hand, GREEN (1986) said: "Alterations in C-band position are observable and can be used judiciously as characters. Until firm homologies can be established for such characters by molecular means, analyses which use them may be open to interpretation, but this does not detract from the value of karyotypic data as alternative and comparative information for systematics". Recently, KING (1991) has published extensive work in support of plylogenetic inferences bases on analyses of C-bands/heterochromatin changes.

7.3. Taxonomical inferences

The usefulness of conventionally stained karyomorphs as taxonomical characters, has been known for some time for Anura species. They supply information of such value, that it has been possible of to distinguish species and genera, as in *Leptodactylus, Adenomera* and *Vanzolinius* (BOGART 1974, HEYER 1974a, 1974b), and has been deemed sufficient to establish primary phylogenetic relationships (BOGART 1972, 1974, MORESCALCHI 1973, GREEN 1986, KING 1990).

The techniques of chromosome banding, of relatively recent application to anuran chromosomes, increase the quantity and quality of information in a high degree (by several magnitude orders) obtained for taxonomical purposes (SCHMID 1978, 1980, GREEN 1986, GREEN & SESSIONS 1991), making greater precision possible in the inferences that are derived from it.

BOGART (1972), BRUM-ZORRILLA & SÁEZ (1973) and FORMAS (1978) reported conventional staining karyomorphs that were found to *B. spinulosus, B. s. spinulosus* and *B. spinulosus* (sic), respectively. The origin of the samples of the former and latter was the province of Mendoza (Argentina) and that of second, the city of La Paz (Bolivia). Among these reports there are discrepancies, being the most notable morphology and centromeric index of some pairs, as well as the presence and location of secondary constrictions (in pair 10 for those of Mendoza-FORMAS, and not found in those of La Paz).

At this comparison level, the findings cited here for the "*spinulosus*" karyomorph, resemble more the Mendoza-FORMAS karyomorph than the La Paz karyomorph, except for the secondary constrictions on the short arm of pair 11. This seems a problem derived from different criteria for processing and ordering the chromosomes used by the authors, and it is possible that both pairs are the same homologue.

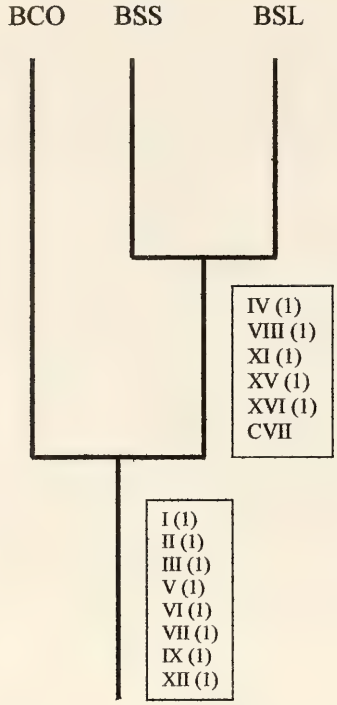

Fig. 12. Cladogram obtained by phylogenetic Hennig 86 software. The only tree produced have a length = 23 and a consitency index (CI = 0.91) and retention index (RI = 0.71). The more basal karyomorph is *BCO ("cophotis"). BSS ("spinulosus")* and *BSL ("limensis")* form a derived cluster. Eight derived characters support the monophyly of the *spinulosus* group and six support the clade *spinulosus-limensis.*

At the level of banding karyomorph comparison, the present results indicate the existence of three karyomorphs in population of the *spinulosus* group: "*spinulosus*", "*limensis*" and "*cophotis*". So there are only three karyomorphs for six nominal entities. An obvious question needs to be asked is in relation to which would be the phylogenetic and taxonomic status of such entities belonging to the *B. spinulosus* group from Peru. The resultant cladograms (fig. 12) are important as a base for future discussion of probable phylogenetic relationships between all members of the *spinulosus* group, but the information obtained is sufficient for some taxonomic reflections and conclusions on the natural populations that inhabit Peru.

Nevertheless to test the validity of the conclusions on the status of the populations, based on the phylogeny of the karyomorphic differences and to consider them sufficient to issue a pronouncement that affects their taxonomy, I believe prudent to reinforce my opinions taking into account complementary observations and data from additional disciplines.

Each karyomorph would correspond to a "good" species. It does not seem to be any doubt in accepting this status for carriers of the "*cophotis*" karyomorph, since *B. cophotis* is considered a species clearly different (phaneric or alomorphic) from the other entities that comprise the *spinulosus* group (Vellard 1959), and no hybrids or

intermediate forms have been found in areas where they are in contact with the "*spinulosus*" karyomorphs.

More difficult seems to accept the condition of full species for "*spinulosus*" and "*limensis*". In the "*spinulosus*" karyomorph four nominal entities of different status are found according to the literature, which I call phenotypes (*spinulosus, arequipensis, flavolineatus* and *trifolium*) following SINSCH (1986). They could not be considered even as subspecies since, in addition to their karyomorphic uniformity, SHERIF (1990, cited by SINSCH 1991) determined the enzymatic equality of 23 loci (all the loci tested) between *trifolium* and *spinulosus*, while MAXSON (1984) found almost impossible to distinguish the set *spinulosus-flavolineatus-trifolium* in Peru in terms of albumin immunological distances (ID). This author reports very low values (between zero and three units ID), that do not indicate a long time of genetic isolation or coalescence [from zero to 0.6 million of years (MY)]. From the osteological point of view, MARTIN (1972) considers very homogeneous thereferred species set. But it is SINSCH (1986) who explains better the idea of not considering to each of these phenotypes as subspecies, by concluding that "... found always within seven populations in reproduction ... two or three phenotypes being reproduced between them. The frequency varied in the different populations, but as the most common form resulted *trifolium* (40–65 %), followed by *spinulosus* (35–50 %) and *flavolineatus* (until 10 %). At present, a separation at subspecies or species level seems doubtful". A similar situation was observed here in the sample from Arequipa where four phenotypes form integrades (the three of SINSCH plus *arequipensis*). For these reasons the carriers of the "*spinulosus*" karyomorph, would constitute a unique but variable or polyphenic species.

The carriers of the "*limensis*" karyomorph possess sufficient attributes as to be considered a full species, accepting the category that BLAIR (1972), MARTIN (1972) and CEI (1972) gave to it. The "*limensis*" karyomorph is stable along its distribution area and no hybrid karyomorphs were found in contact zones with other *Bufo* karyomorphs, as in the locality of Trujillo (La Libertad), where it coexists with *B. marinus poepigii,* as well as in Canta (Lima) and Camaná (Arequipa), where it interacts with the "*spinulosus*" karyomorph. MARTIN (1972) considers *B. limensis* osteologically different from the rest of the members of the *spinulosus* group, while MAXSON (1984) indicates that *limensis* has a considerable ID (27 units), respect to the set *spinulosus-flavolineatus-trifolium,* which corresponds to a genetic isolation or coalescence that would be between 14–16 MY. Furthermore, *limensis* differs ecologically from the other representatives of group. It is a form relatively more adapted to severe xeric conditions, as the Peruvian coastal desert, that presents scarce annual rainfall [less than 30 mm per year (TOSI 1960)].

Complementary information (G. YBAZETA & J. H. CÓRDOVA unpublished data) show that *spinulosus* and *limensis* have notable and constant differences in the total protein pattern and in esterase allozymes of its parotoid poisons. Twelve over 42 (28.57 %) protein band differences are found between them, while three esterases systems (Est – I, Est – II and Est – III) characterize the poison of *spinulosus; limensis* poison exhibits only one (Est – II). On the other hand, the *limensis* tadpole differs from *spinulosus* in some morphometric characters and in the arrangement of denticules of the oral disk (A. ANGULO, in preparation).

A related form to *B. limensis,* designated *B. limensis vellardi* by LEVITON & DUELLMAN (1978) (= *B. spinulosus orientalis* Vellard, 1959) and later *B. vellardi* by

Duellman & Schulte (1992), has been reported for the upper Marañón river. According to Leviton & Duellman (1978), this is a very little studied form, and it is not known if it constitutes an isolated population or if still it maintains gene flow with *limensis* of the North Coast through the dry corridor of the Huancabamba depression.

7.4. Proposal of classification

According to the cladistic analysis made from their karyomorphs and the complementary data found, the following natural taxonomical rearragenment for the *spinulosus* species group from Peru is suggested:

Bufo spinulosus group, constituted by four species:

a) *Bufo spinulosus* Wiegmann, 1834, that includes *B. arequipensis* Vellard, 1959, *B. flavolineatus* Vellard, 1959 and *B. trifolium* (Tschudi, 1845), considered only as variant phenotypes of *B. spinulosus;*

b) *B. limensis* Werner, 1901 (probably includes *B. vellardi* Leviton & Duellman, 1978),

c) *B. cophotis* Boulenger, 1900 and

d) *B. corynetes* Duellman & Ochoa, 1991 (no samples examined in this study).

The next step will be to asertain the banding karyomorphs of *B. vellardi* and *B. corynetes,* as well as the populations of the *spinulosus* group that are found outside Peru, in order to define their taxonomic status within the classificatory context proposed here.

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